



TITLE:

FGF-2 Stimulates the Growth of Tenogenic Progenitor Cells to Facilitate the Generation of Tenomodulin-Positive Tenocytes in a Rat Rotator Cuff Healing Model.

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- 1 **Title:** FGF-2 Stimulates the Growth of Tenogenic Progenitor Cells to Facilitate the Generation of
- 2 *Tenomodulin*-positive Tenocytes in a Rat Rotator Cuff Healing Model
- 3
- 4 **Running title:** Effect of FGF-2 on Rotator Cuff Healing
- 5
- 6

7 Abstract

8 **Background:** Fibroblast growth factor (FGF)-2 has the potential to enhance tendon-to-bone healing
9 after rotator cuff (RC) injury.

10 **Hypothesis:** FGF-2 stimulates tenogenic differentiation of progenitors to improve the biomechanical
11 strength and histological appearance of repaired RCs in rats.

12 **Study Design:** Controlled laboratory study.

13 **Methods:** Adult male Sprague-Dawley rats (n = 156) underwent unilateral surgery to repair the
14 supraspinatus tendon to insertion sites. The FGF-2-treated (gelatin hydrogel containing 5 µg of
15 FGF-2) and control groups (gelatin hydrogel only) were compared to investigate the effects of FGF-2
16 at 2, 4, 6, 8, and 12 weeks postoperatively. Biomechanical testing was performed at 6 and 12 weeks.
17 Semi-quantitative histological analysis and immunohistochemistry for the proliferating cell nuclear
18 antigen (PCNA) was performed, and the expression of tendon-related markers, including *Scleraxis*
19 (*Scx*) and *Tenomodulin* (*Tnmd*) were monitored by real-time reverse transcription polymerase chain
20 reaction (RT-PCR) and *in situ* hybridization. SRY-box containing gene 9 (Sox9) expression was
21 monitored by RT-PCR and immunohistochemistry. At 2 and 4 weeks, immunohistochemistry for
22 mesenchymal stem cell (MSC) markers were also performed.

23 **Results:** The FGF-2-treated group demonstrated a significant improvement in mechanical strength at
24 6 and 12 weeks and significantly higher histological scores than the control at ≥ 4 weeks. The average
25 incidence of PCNA-positive cells was significantly higher at 2 and 4 weeks, and more cells

26 expressing MSC markers were detected at the insertion site in the FGF-2-treated group. The
27 expression level of *Scx* increased significantly in the FGF-2-treated group from 4 to 8 weeks, while
28 the *Tnmd* level increased significantly from 4 to 12 weeks postoperatively. The localization of *Tnmd*
29 overlapped with the locations of reparative tissues accompanying collagen fibers with an aligned
30 orientation. The *Sox9* expression was significantly upregulated at 4 weeks in the FGF-2-treated
31 group.

32 **Conclusion:** FGF-2 promotes growth of the tenogenic progenitor cells, which participates in
33 tendon-to-bone healing, resulting in the biomechanical and histological improvement of repaired RC.

34 **Clinical Relevance:** These findings provide clues on the clinical development of regenerative repair
35 strategies for RC injury.

36 **Key Terms:** FGF-2, rotator cuff healing, *Scleraxis*, *Tenomodulin*, *Sox9*, cell proliferation, tenogenic
37 progenitors

38

39 **What is known about the subject:**

40 Rotator cuff (RC) tears are a common cause of shoulder pain and dysfunction that often require
41 surgical repair. A relatively high repair failure rate (21.7%, 187/861 patients) was reported in a recent
42 meta-analysis of 14 studies after RC surgical repair.¹⁴ Various efforts (e.g., the application of growth
43 factors or transplantation of mesenchymal stem cells) have been made to promote a reparative
44 response.^{7,8,9,24,28} Administration of the fibroblast growth factor 2 (FGF-2) has the potential to
45 enhance the reparative response during the RC healing process;⁹ however, little is known about the
46 biological mechanism of the repair-promoting effect (improvement of biomechanical strength or
47 histological appearance) by FGF-2 during RC healing.

48
49 **What this study adds to existing knowledge:**

50 In a rat model, the administration of FGF-2 to the healing tendon-bone interface after surgical
51 repair of an acute supraspinatus injury improved the mechanical strength and histological appearance
52 between the tendon and bone at ≥ 4 weeks. Moreover, the proliferation of cells expressing markers of
53 mesenchymal stem cells was stimulated in the FGF-2-treated group during the early phase of RC
54 healing. Subsequently, upregulation of the tendon markers such as *Scleraxis* (*Scx*) and *Tenomodulin*
55 (*Tnmd*) were detected in the FGF-2-treated group at ≥ 4 weeks. Interestingly, localization of *Tnmd*
56 mRNA overlapped with the locations of reparative tissues accompanying collagen fibers with an
57 aligned orientation during RC healing.

58 This suggests that FGF-2 promotes the growth of tenogenic progenitor cells to participate in

59 tendon-to-bone healing, resulting in the histological and biomechanical improvement of RC healing.

60

61 Introduction

62 A rotator cuff (RC) injury is one of the most common causes of shoulder pain and dysfunction,
63 often requiring surgical repair. Despite great improvement in surgical techniques for repairing RC
64 tears, a recent meta-analysis of 14 studies reported on an imaging assessment of the structural
65 integrity of RC repair, and it demonstrated a high rate of failed repair (21.7 %, 187/861 patients) at
66 the latest follow-up.¹⁴ Therefore, developing new strategies for enhancing tendon-to-bone healing is
67 imperative.

68 Various growth factors are endogenously expressed during tendon-to-bone healing, suggesting that
69 they play critical roles in the repair process,²⁷ and there is an interest regarding their use as
70 therapeutic agents for enhancing RC repairs. Of those, fibroblast growth factor-2 (FGF-2) is a potent
71 mitogen for a wide variety of cells, including mesenchymal stem cells (MSCs) and progenitor cells.^{11,}
72 ^{19, 25} We previously demonstrated that the local administration of FGF-2 accelerates the initial
73 tendon-to-bone healing as indicated by biomechanical tests and histological analyses after
74 supraspinatus tendon repair in rats.⁹ However, the biological mechanism of the reparative
75 enhancement by FGF-2 during RC healing is unclear.

76 Recently, several genes have been identified as specific tendon markers. Scleraxis (Scx) is a basic
77 helix-loop-helix transcription factor that is expressed in both tenogenic progenitors and tenocytes.^{13,}
78 ¹⁵ Tenomodulin (Tnmd) is a type II transmembrane protein that is specifically expressed in the dense
79 connective tissues such as tendons and ligaments.^{17, 18} Tnmd is detected in mature tenocytes and is

80 positively regulated by *Scx*.^{3, 18} Identification of these marker genes enables us to study the cellular
81 and molecular events that occur during the establishment of the osteotendinous junction in detail.
82 Recently, we reported that a subset of *Scx*-positive progenitors with an expression history of *SRY-box*
83 *containing gene 9* (*Sox9*) contributes to the formation of enthesis during development.²⁰
84 The purpose of this study was to investigate the effects of FGF-2 administration on cell
85 proliferation and the expression of enthesis-related marker genes (i.e., *Scx*, *Sox9*, and *Tnmd*) during
86 RC tendon-to-bone healing in rats. The effects on biomechanical strength and histological
87 appearance during healing were also investigated. We hypothesized that FGF-2 stimulates the
88 tenogenic differentiation of progenitors and improves the biomechanical strength and histological
89 appearance of repaired RCs in rats.
90

91 **Materials and Methods**

92 **Study Design**

93 This study was approved by our institution's Animal Studies Committee and the Institutional
94 Animal Care and Use Committee. We used a rat RC healing model, in which the supraspinatus
95 tendon insertion site was transected and surgically repaired immediately, to investigate the effects of
96 FGF-2 on tendon-to-bone healing. In this model, a normal tendon-to-bone insertion site is not
97 regenerated even after 16 weeks postoperatively.²³

98 We included 156 adult male Sprague-Dawley rats (19–21 weeks old; mean weight, 475 ± 37 g)
99 that underwent unilateral surgery for supraspinatus tendon repair immediately after transaction, and
100 they were randomly allocated to the control or FGF-2-treated groups ($n = 78$ per group). In the
101 control, gelatin hydrogel sheets (MedGEL Co., Kyoto, Japan) with a phosphate-buffered saline
102 (PBS) were applied between the supraspinatus tendon and bone. In the FGF-2-treated group, gelatin
103 hydrogel sheets containing 5 μ g of rhFGF-2 (Kaken Pharmaceutical Co., Tokyo, Japan) were applied.
104 All rats were sacrificed at 2, 4, 6, 8, or 12 weeks postoperatively. At each time point, 6 specimens per
105 group were used for histological evaluation (i.e., histology, immunohistochemistry, or *in situ*
106 hybridization), and 6 per group were analyzed with real-time reverse transcription polymerase chain
107 reaction (RT-PCR). At 6 and 12 weeks, 9 specimens per group were used for biomechanical testing.

108

109 **Local Administration of FGF-2**

110 We used a biodegradable gelatin hydrogel sheet²¹ (MedGel PI5, MedGEL Co) as a carrier for
111 the FGF-2. In our pilot study, three doses of FGF-2 (0.5, 5, and 50 $\mu\text{g}/\text{site}$) in gelatin hydrogel sheets
112 were compared between the carrier-only and suture-only groups using the same method in the
113 present study ($n = 6$ per group underwent histological and biomechanical testing). At 12 weeks
114 postoperatively, a significant improvement in the ultimate load-to-failure was achieved in all
115 FGF-2-treated groups (0.5- μg , 29.1 ± 10.1 N; 5- μg , 31.7 ± 8.5 N; and 50- μg , 25.9 ± 8.6 N) compared
116 to the carrier-only (15.8 ± 4.5 N) and suture-only groups (12.4 ± 2.4 N). The histological scores in
117 the FGF-2-treated groups (0.5- μg , 9.7 ± 1.0 ; 5- μg , 10.7 ± 0.5 ; and 50- μg , 10.3 ± 0.5) were higher
118 than that in the suture-only (7.5 ± 0.5) and carrier-only groups (7.8 ± 0.8). Although there were no
119 significant differences among the three FGF-2-treated groups, we used the 5- μg dose of FGF-2,
120 because the 5- μg FGF-2-treated group showed the highest histological score and the ultimate
121 load-to-failure.

122 Before administration, freeze-dried gelatin hydrogel sheets (1 mg) for each shoulder were soaked
123 with 10 μL of a PBS or FGF-2 (500 $\mu\text{g}/\text{mL}$), and the sheets were incubated for 60 min at room
124 temperature (RT).

125

126 **Surgical Procedure**

127 The rats underwent left shoulder surgery, as previously described.^{6, 23} Briefly, after the
128 administration of general anesthesia with an intraperitoneal injection of pentobarbital (25–30 mg/kg),

129 longitudinal antero-lateral skin and deltoid muscle incisions were made, and the supraspinatus
130 tendon was exposure. The supraspinatus tendon was detached sharply at its insertion (3 mm in the
131 anterior-to-posterior dimension), and the fibrocartilaginous stump on the greater tuberosity was
132 completely removed using a high-speed bur until bleeding was observed. A 0.8-mm drill hole was
133 created transversely in an anterior-posterior orientation through the proximal part of the humerus. We
134 sutured the torn supraspinatus tendon to its anatomic position using a double-armed 5-0 prolene
135 suture (Ethicon, Somerville, NJ) using a modified Mason-Allen method. A gelatin hydrogel sheet
136 soaked with PBS or FGF-2 was fixed to the torn end of the tendon using a 5-0 prolene suture, which
137 is used for repair, and it was secured between the tendon and bone. The incision was closed in layers
138 with 4-0 nylon sutures. All rats were permitted cage activities without immobilization
139 postoperatively.

140

141 **Biomechanical Testing**

142 As previously described,^{9, 26} 9 animals per group were tested at 6 and 12 weeks postoperatively,
143 and 9 intact shoulders were used as a normal control. All specimens were frozen at -80°C until
144 testing. After thawing, the supraspinatus tendon repair to the humerus was dissected from the
145 surrounding tissues with a custom-designed double scalpel blade to a 2-mm-width to standardize the
146 tendon tested, and the supraspinatus muscle belly and any scar tissue was removed. Each specimen
147 was preloaded to 0.1 N and loaded to failure with a conventional tensile tester (STA1225; Orientec,

148 Tokyo, Japan) at a rate of 10 mm/min, and the ultimate load-to-failure and the failure site were
149 recorded. The linear region of the load-displacement curve was used to calculate the stiffness. The
150 cross-sectional area of the repaired insertion site, including its width and thickness, was measured
151 using a digital micro-caliper.⁷ The ultimate load-to-failure was divided by the cross-sectional area of
152 the repair site to determine the ultimate stress-to-failure.

153

154 **Histological Evaluation**

155 The tissues were fixed overnight in 4% paraformaldehyde at an abduction angle of 30° and were
156 decalcified in Morse's solution (10% sodium citrate and 22.5% formic acid). After decalcification,
157 the tissues were dehydrated and were embedded in paraffin. The sections were viewed under an
158 Olympus BX-51 microscope (Olympus Optical, Tokyo, Japan), and digital images were taken using
159 a DP70 camera (Olympus Optical). To evaluate healing at the tendon-to-bone site semi-quantitatively,
160 we used a computerized image analysis (Image J, National Institutes of Health, Bethesda, MD) and a
161 modified scoring system (Table 1) based on previously reported histological evaluations.^{24, 26, 28} We
162 scored the healing insertion site according to three histological parameters (i.e., cellularity,
163 vascularity, and collagen fiber orientation). Three sequential sections were obtained from each
164 animal for each staining, and the results were averaged.

165 To analyze cellularity, all cell nuclei in a randomly selected region of interest ($200 \times 200 \mu\text{m}$) were
166 counted to calculate the number per mm^2 at the insertion site (100–400 μm proximal to the bone
167 trough) on hematoxylin and eosin (HE)-stained slides under 100-fold magnification.

168 To analyze vascularity, all blood vessels were counted to calculate the mean vessel number from
169 three sequential sections at the insertion site (100–400 μm proximal to the bone trough) on
170 HE-stained slides under 100-fold magnification.

171 The collagen fiber orientation was assessed by picrosirius-red stained sections using a polarizing
172 microscope.^{7, 26} On eight-bit digitized images, intense white areas of brightly diffracted light on a
173 gray scale (black, 0; white, 255), were observed. Higher gray scales indicated more organized
174 collagen fibers. We randomly selected 10 square areas ($100 \times 100 \mu\text{m}$) at the insertion site (100–400
175 μm proximal to the bone trough) under 12.5-fold magnification, and gray scales were measured
176 using Image J. The values from the 10 areas were averaged to obtain a value of average gray scales
177 per specimen. To reduce sampling errors, we took photomicrographs of three sequential sections
178 from each specimen under the same illumination conditions.

179 Cellularity and collagen fiber orientation were scored using the percentage of the value measured
180 in six right shoulders (intact side). A perfect score is 12 points (Table 1). Analysis was performed by
181 two colleagues who were blinded to the groups for each specimen and the time points throughout the
182 histological examinations.

183

184 **Immunohistochemistry**

185 Semi-serial sections (4 μ m) were prepared for immunostaining. To assess the cell proliferation, the
186 expression of Sox9 and existence of MSCs, we performed immunostaining for PCNA and Sox9 at all
187 time points, MSC-related surface antigens (CD105, CD90, and CD73), and hematopoietic cell
188 antigen (CD45 and CD34) at 2 and 4 weeks postoperatively.²

189 The sections were deparaffinized; rehydrated; and incubated overnight at 4°C with a primary
190 antibody recognizing PCNA (dilution 1:200, clone PC10; Dakopatts, Copenhagen, Denmark), Sox9
191 (1:200, AB5535; Millipore, Billerica, MA), CD105 (1:50, #05-1424; Millipore), CD90 (1:200,
192 AB225; Abcam, Cambridge, MA), CD73 (1:200, AB175396; Abcam), CD45 (1:1000, AB10558;
193 Abcam), and CD34 (1:100, AB 64480; Abcam). Subsequent reactions were performed using a
194 peroxidase-labeled antibody (Histofine Simple Stain Max PO; Nichirei, Tokyo, Japan), which
195 reacted with a 3, 3-diaminobenzidine solution and were counterstained in 0.5% Mayer's
196 hematoxylin.

197

198 **Estimation of Cell Proliferation and the Expression of Sox9 at the Healing Site**

199 To estimate cell proliferation at the healing site, we modified the previously described method for
200 PCNA-positive cell counting.¹² Three square areas (200 \times 200 μ m) at the insertion site (100–400 μ m
201 proximal to the bone trough) were selected randomly under 200-fold magnification. The percentage

202 of PCNA-positive cells was calculated by dividing the number of PCNA-positive cells by the total
203 number of cells in the area; the average percentage of three areas from each slide was recorded.

204 The percentage of Sox9-positive cells was calculated in the same manner.

205

206 **RNA Isolation and Real-time Reverse Transcription Polymerase Chain Reaction Analysis**

207 At all time points, the left (operative side) and right (intact side) tendon-to-bone tissues were
208 dissected and flash frozen in liquid nitrogen and crushed using a Multi-beads Shocker (Yasui Kikai,
209 Osaka, Japan). After homogenization, total RNA was extracted and purified per the manufacturer's
210 instructions using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA). The total RNA
211 (1 µg) was reverse transcribed to complementary DNA (cDNA) using the Thermoscript RT kit
212 (Invitrogen). Real-time PCR was performed using an ABI7500 system (Applied Biosystems, Foster,
213 CA) with TaqMan Universal PCR Master Mix and TaqMan™ Gene Expression Assays for the
214 following genes: *Scx* (Assay ID: Rn01504576_m1), *Tnmd* (Rn00574164_m1), *Sox9*
215 (Rn01751069_mH), and *18S ribosomal RNA* (Rn03928990_g1). Each sample was analyzed in
216 duplicate. The relative expression levels for the target genes were analyzed using the comparative Ct
217 method. The delta-Ct values were calculated by the difference between the Ct values of the target
218 genes and *18S ribosomal RNA*. The results are represented as the relative gene expression compared
219 to the intact tendon.¹⁶

220

221 ***In situ* Hybridization**

222 *In situ* hybridization was performed to detect the expression of *Scx* and *Tnmd* mRNA as previously
223 described.¹ Briefly, 5- μ m sections were deparaffinized, rehydrated, incubated with proteinase K (20
224 μ g/mL; Gibco BRL, Rockville, MO), post-fixed in 4% paraformaldehyde, acetylated with 0.1 M
225 triethanolamine containing 0.25% acetic anhydride for 10 min, dehydrated, and air-dried.

226 Sense and antisense RNA probes labeled with digoxigenin (DIG) for *Scx* or *Tnmd* were
227 synthesized by *in vitro* transcription with a DIG RNA labeling kit (Roche, Mannheim, Germany)
228 using the following cDNA clones: rat *Scx* cDNA containing a 0.726 kb fragment was subcloned into
229 pBSII SK+ (Stratagene, La Jolla, CA) and rat *Tnmd* cDNA containing a 0.607 kb fragment was
230 subcloned into pCRII-TOPO (Invitrogen), as a template after linearization. Hybridization was
231 performed at 50°C for 16 h. After hybridization, the sections were washed in $2 \times$ SSC containing
232 50% formamide, treated with RNase A (20 μ g/mL), and washed in $2 \times$ SSC. Immunological probe
233 detection was performed with an anti-DIG-AP Fab fragment (Roche) and BM Purple (Roche). The
234 specificity of these antisense probes was confirmed on mouse embryo (E16.5) sections as positive
235 controls. Sense probes for *Scx* and *Tnmd* were used on sections as negative controls.

236

237 **Evaluation of the Association between the Locations of Reparative Tissue Accompanying** 238 **Collagen Fibers with an Aligned Orientation and *Scx* or *Tnmd* mRNA**

239 To investigate the correlation between the location of healing tissues with aligned collagen fiber
240 orientation and the localization of *Scx* or *Tnmd* mRNA, images from picrosirius-red staining and *in*
241 *situ* hybridization in sections in the treatment and control groups at 8 weeks postoperatively (n = 12)
242 were evaluated, and nine areas (tendon, suture, and insertion areas of the articular side, middle, and
243 bursal side) were set (see Figure 5C). Collagen fiber orientation for each area was evaluated by
244 applying the gray scale as described above. Expression levels of *Scx* or *Tnmd* mRNA (density of
245 hybridization signals) in each area were graded using previously reported²³ standards to define high
246 (4), moderate (3), low (2), and undetectable (1) levels, and the highest grade for each area from each
247 specimen was recorded.

248

249 **Statistical Analysis**

250 Before the study initiation, a power analysis was performed using the G*Power 3.1 program,⁵
251 which we used in our pilot study described above that had a similar rat model. A minimum of 9
252 specimens per group for each time point was required for biomechanical testing (Cohen's d, ≥ 1.45),
253 and a minimum of 6 specimens per group for each time point was required for histological evaluation
254 (Cohen's d, ≥ 2.04), with an α value of 0.05 and a power of 0.80 based on the effect size. The sample
255 size for real-time RT-PCR was determined according to the power analysis for histological
256 evaluation. Our primary analysis compared measurements between the groups at each time point,
257 and the secondary analysis compared measurements within the groups over time. We used

258 nonparametric statistical methods for all the analyses, because the data were non-normally
259 distributed. Statistical significance between the groups at each time point was estimated using the
260 Mann-Whitney U test. Differences in the measurements within groups over time were analyzed using
261 the Kruskal-Wallis test, followed by the post-hoc Scheffe test for multiple comparisons.
262 The correlation between the collagen fiber orientation and the expression levels of *Scx* or *Tnmd*
263 mRNA were assessed using Spearman's correlation analysis. Values of $P < .05$ were considered
264 statistically significant.
265

266 **Results**

267 **Biomechanical Testing**

268 Three of 9 specimens from the normal shoulders failed at the insertion site; the other 6 failed at the
269 tendon mid-substance. All specimens from the operated groups failed at the site of surgical repair
270 (Table 2).

271 At 6 weeks postoperatively, the ultimate load-to-failure, stiffness, and ultimate stress-to-failure
272 were significantly higher in the FGF-2-treated group than in the control. No significant differences
273 were observed between the groups in the cross-sectional area of the repair site.

274 At 12 weeks postoperatively, the ultimate load-to-failure and ultimate stress-to-failure were
275 significantly higher in the FGF-2-treated group than in the control. No significant differences were
276 observed between the groups in the stiffness and cross-sectional area of the repair site.

277

278 **Histological Analysis**

279 **Effects of FGF-2 on the histological appearance**

280 At 2 weeks postoperatively, hyper-cellular and hyper-vascular granulations were noted in the
281 insertion site (Figure 1A, G), and areas of brightly diffracted polarized light were rarely observed at
282 the insertion site in any of the groups (Figure 1D, J).

283 From 6 to 12 weeks, although granulations decreased compared to the previous time point, the
284 tendon-to-bone area remained hyper-vascular and filled with disorganized collagen fibers in the

controls (Figure 1B, C, E, F). Conversely, in the FGF-2-treated groups, these granulations were decreased, and the area of brightly diffracted polarized light at the insertion site was larger than that in the controls (Figure 1H, I, K, L). The histological differences between the treatment and control group were observed at ≥ 4 weeks.

At the bone surface, normal fibrocartilaginous entheses was not regenerated in any specimen from any group at any time point.

In both groups, cellularity and vascularity decreased, but the collagen fiber orientation and total histological scores increased with time (Table 3). The mean number of blood vessels at the insertion site was significantly smaller in the FGF-2-treated group than in the control at 8 and 12 weeks postoperatively. Collagen fiber orientation and the total score were significantly higher in the FGF-2-treated group than in the control at ≥ 4 weeks.

296

297 **Effects of FGF-2 on cell proliferation**

At 2 and 4 weeks postoperatively, greater numbers of PCNA-positive cells were observed in the granulation tissue between the tendon and bone in the FGF-2-treated group than those in the control (Figure 2A, C). At ≥ 6 weeks, PCNA-positive cells obviously decreased and were mainly present at the bone surface in both groups (Figure 2E). The percentage of PCNA-positive cells was significantly larger in the FGF-2-treated group than in the control at 2 weeks ($45.4 \pm 9.3\%$ vs. $30.8 \pm 5.7\%$; $P = .016$) and 4 weeks ($41.9 \pm 8.0\%$ vs. $29.0 \pm 7.4\%$; $P = .025$). There were no differences

304 between the groups at 6 weeks (control, $21.2 \pm 4.6\%$; FGF-2-treated, $23.8 \pm 5.3\%$; $P = .423$), 8
305 weeks (control, $19.7 \pm 2.7\%$; FGF-2-treated, $22.3 \pm 5.7\%$; $P = .263$) and 12 weeks (control, $19.0 \pm$
306 3.8% ; FGF-2-treated, $19.6 \pm 5.3\%$; $P = .749$) (Figure 2E).

307

308 **Effect of FGF-2 on the expression of *Scx* and *Tnmd***

309 Real-time RT-PCR analysis showed that a significantly higher expression of *Scx* was detected in
310 the FGF-2-treated group than in the control at 4, 6, and 8 weeks ($P = .010$, $.010$, and $.004$,
311 respectively). There were no differences between the control and FGF-2 groups at 2 and 12 weeks (P
312 $= .631$, and $.150$, respectively) (Figure 3A). *Tnmd* expression was significantly higher in the
313 FGF-2-treated group than in the control at 4, 6, 8, and 12 weeks ($P = .004$, $.010$, $.004$, and $.016$,
314 respectively). There were no differences between the groups at 2 weeks ($P = .631$) (Figure 3B). *Scx*
315 expression was transiently upregulated from 4 to 8 weeks; meanwhile *Tnmd* expression was
316 persistently upregulated by FGF-2 treatment even after 12 weeks postoperatively (Figure 3).

317 We then performed *in situ* hybridization to analyze the localization of *Scx* and *Tnmd* mRNA. In
318 both groups, hybridization signals for *Scx* were detected in the spindle-shaped cells at the tendon
319 mid-substance at all time points (Figure 4A, C, I, K), and a few hybridization signals for *Scx* were
320 detected in the round and spindle-shaped cells within the granulation tissue around the suture at 4, 6,
321 and 8 weeks (data not shown). In the control, hybridization signals for *Scx* were rarely detected
322 within the fibro-vascular tissue at the bone surface at any time points (Figure 4E, G). However, in the

323 FGF-2-treated group, *Scx* were expressed in the spindle-shaped cells at the bone surface at ≥ 4 weeks
324 (Figure 4M, O).

325 *Tnmd* signals were also detected in the spindle-shaped cells at the tendon mid-substance in both
326 groups at all time points, and hybridization signals increased with time (Figure 4B, D, J, L). In the
327 FGF-2-treated group, the distribution of these signals expanded into reparative tissues near the
328 suturing site compared to the control. However, *Tnmd* was rarely detected at the bone surface in
329 either group at any time point (Figure 4F, H, N, P).

330

331 **Correlation between the locations of healing tissue with aligned collagen fiber orientation and** 332 ***Scx* or *Tnmd* mRNA**

333 We assessed 108 areas in both groups at 8 weeks postoperatively (Figure 5). A weak positive
334 correlation between the location of aligned collagen fiber orientation and expression levels of *Scx*
335 mRNA in each area was observed (Spearman's rank correlation [ρ] = .27, $P < .001$) (Figure 5D).
336 Meanwhile, a strong positive correlation between the location of aligned collagen fiber orientation
337 and the expression levels of *Tnmd* mRNA in each area was observed ($\rho = .88$, $P < .001$) (Figure 5E).

338

339 **Effect of FGF-2 on Sox9 expression**

340 Real-time RT-PCR analysis showed that *Sox9* expression was significantly higher in the
341 FGF-2-treated group than in the control at 4 weeks ($P = .004$). However, at 2, 6, 8 and 12 weeks,

there were no statistically significant differences between the groups ($P = .337$, $.749$, $.200$ and, $.337$, respectively) (Figure 6).

In immunostaining for Sox9 (Figure 7), Sox9-positive cells were mainly observed at the tendon-to-bone insertion site, but a few Sox9-positive spindle-shaped cells were also detected at the tendon mid-substance at ≥ 4 weeks in both groups. The percentage of Sox9-positive cells was significantly higher in the FGF-2-treated group than in the control at 4 weeks ($23.7 \pm 4.9\%$ vs. $14.0 \pm 3.6\%$; $P = .010$). There were no differences between the groups at 2 weeks (control, $10.1 \pm 4.8\%$; FGF-2-treated, $13.4 \pm 6.6\%$; $P = .337$), 6 weeks (control, $19.7 \pm 2.7\%$; FGF-2-treated, $22.3 \pm 5.7\%$; $P = .263$), 8 weeks (control, $10.3 \pm 1.1\%$; FGF-2-treated, $11.2 \pm 1.5\%$; $P = .423$), and 12 weeks (control, $10.1 \pm 2.6\%$; FGF-2-treated, $11.3 \pm 3.4\%$; $P = .749$) (Figure 7I).

352

Effect of FGF-2 on mesenchymal stem cells marker-positive cells in the early phase of healing

To analyze MSCs, serial sections were immunostained for MSC-related surface antigens (CD105, CD90, and CD73) and hematopoietic cell antigen (CD45 and CD34) at 2 and 4 weeks postoperatively. CD45- and CD34-positive cells were rarely detectable at the insertion site in either group. In the FGF-2-treated group, cells positive for CD105, CD90, and CD73 were detected more within the reparative tissue at the insertion site than in the control (Figure 8).

359 Discussion

360 Our findings support our hypothesis that FGF-2 stimulates the tenogenic differentiation of
361 progenitors to improve the biomechanical strength and histological appearance of repaired RCs in
362 rats. Previous studies^{6, 23} have described the healing response as observed using histological
363 evaluation in a rat RC acute injury and immediate repair model, and the healing response occurred
364 almost exclusively within the tendon-to-bone insertion site that was filled with hyper-cellular and
365 hyper-vascular granulation tissues by postoperative day 10. As healing progresses, cellularity and
366 vascularity decrease, and then the improvement of collagen fiber orientation and fiber integration
367 into the bone were observed to some extent. However, a histologically normal tissue is not
368 regenerated. Instead, the disorganized fibro-vascular scar interposes at the insertion site without
369 supplementation of growth/differentiation factors.²³ Without FGF-2 administration, in this study, the
370 fibro-vascular scar interposes between the tendon and bone even 12 weeks after RC repair surgery.
371 In contrast, the administration of FGF-2 clearly decreased the occurrence of fibro-vascular scarring
372 at the insertion site and increased the aligned orientation of collagen fibers at ≥ 4 weeks, as indicated
373 by significantly higher histological scores (Figure 1, Table 3). Moreover, even though normal
374 biomechanical strength was not achieved at 12 weeks postoperatively (ultimate load-to-failure,
375 56.7% of normal controls; stiffness, 33.3% of normal controls; ultimate load-to-stress, 35.1% of
376 normal controls), FGF-2 administration significantly increased the biomechanical strength by 6
377 weeks postoperatively (Table 2). In an ovine study model on tendon-to-bone healing, the

378 disorganized fibro-vascular scar at the insertion site was weaker than the axially-aligned collagen
379 fibers in the bone.⁸ These results suggest that the improvement of mechanical strength by FGF-2
380 treatment is associated with decreases in the fibro-vascular scarring and improvement in the collagen
381 fiber orientation at the insertion site.

382 Accumulating evidence suggests that *Scx*-expressing tenogenic cells can participate in a
383 regenerative repair of tendons to promote the formation of collagen fibers with an aligned orientation,
384 which have better tensile strength. Gulotta et al.⁷ demonstrated that application of bone
385 marrow-derived MSCs transduced with *Scx* resulted in an improvement in histological and
386 biomechanical properties at 4 weeks postoperatively in a similar rat RC healing model. Tan et al.²²
387 also reported that transplantation of *Scx*-transduced tendon-derived stem cells provided better tendon
388 repair than that of mock-transduced cells in a rat patellar tendon injury model from the histological
389 and biomechanical viewpoints.

390 Our study showed that FGF-2 administration induced *Scx* expression in the cells of healing tissue
391 4–8 weeks postoperatively, indicating that more tenogenic progenitor cells were generated at the
392 healing sites from the mid-to-late stages of healing (Figures 3–4). During differentiation from
393 tenogenic progenitors to mature tenocytes, the *Scx* expression declines, while *Tnmd* is continuously
394 expressed.^{10, 18} In murine tendon development, *Scx* expression increases from E10.5 to E12.5 and
395 decreases at E13.5, meanwhile the expression of *Tnmd* sharply increases from E13.5 and persists
396 until the late stage in the forelimb tendon cells.¹⁰ These temporal expression patterns of *Scx* and

397 *Tnmd* were also demonstrated in mouse patellar healing.¹⁶ In this study, *Tnmd*-positive tenocyte-like
398 cells were persistently observed until the later stage of healing from 4–12 weeks postoperatively.
399 Interestingly, *Tnmd*-positive tenocyte-like cells were only observed in the area with highly oriented
400 collagen fibers. In particular, these cells were distributed into the reparative tissues near the suturing
401 site in the FGF-2-treated group 4–12 weeks postoperatively, which was consistent with highly
402 oriented collagen fiber areas as shown by the histological evaluation in this study (Figure 5E). These
403 spatial patterns of *Tnmd* expression are similar to that in the late-stage of tendon development,¹⁸
404 suggesting that *Tnmd* is a useful marker for evaluating the differentiation of mature tenocyte-like
405 cells in tendon healing.

406 Despite enhanced differentiation of *Tnmd*-positive tenocyte-like cells, it is unlikely that FGF-2
407 directly stimulated tenogenic differentiation of cells in this later phase of RC healing, since FGF-2 is
408 known to be released from gelatin hydrogel sheets within 2 weeks.²¹ Thus, the FGF-2-stimulated
409 tenogenic repair responses in this study must be primarily mediated by the growth stimulation of
410 undifferentiated mesenchymal cells, as indicated by a higher incidence of PCNA-positive cells at the
411 repair sites during the early phase (Figure 2). In a mouse patellar tendon healing model,⁴
412 undifferentiated mesenchymal progenitors migrate from a paratenon to the healing site by 1 week
413 post-injury. Subsequently, *Scx*-expressing cells appear at ≥ 2 weeks, suggesting that the initial growth
414 of undifferentiated mesenchymal progenitors have a critical role for the subsequent tenogenic
415 differentiation of cells during the later phase of healing. In agreement with the previous *in vitro*

416 studies^{11, 19, 25} that have shown that FGF-2 potentially stimulates the self-renewal of MSCs and/or
417 progenitor cells without a loss of potentials for the subsequent cell differentiation, we demonstrated
418 at least in the present study that the healing process started with a better accumulation of cells
419 expressing MSC-related markers within 2 weeks in the FGF-2-treated group (Figure 8).

420 There are several limitations to the current study. First, our acutely created tear and immediate
421 repair model does not reflect chronic degenerative RC tears, and our findings in this rat model may
422 not directly translate to humans, because the healing processes in healthy animals may not mirror the
423 situations encountered in clinical practice. However, based on anatomical similarities between
424 humans, this model has been widely used to investigate the mechanism of healing^{6, 23, 27} and the
425 method of healing enhancement.^{7, 9, 24} Second, this study did not directly identify the function of
426 tenogenic marker genes during the RC healing process. Further studies using a knockout model on
427 *Scx* or *Tnmd* are required to determine the roles of these genes during the RC healing process. Third,
428 our findings demonstrated that the biomechanical and histological values of the control group did not
429 reach those of the FGF-2-treated group by 12 weeks. A longer time period may be necessary to
430 understand whether the effect observed in the FGF-2-treated group will persist throughout the
431 healing response. Fourth, the sample size, which was based on the large effect size, was relatively
432 small, and it may be underpowered to detect significant differences. Likewise, our histological
433 evaluation may also be underpowered, because it did not include all sections from the specimens.
434 However, there was little or no discrepancy among our findings observed in various evaluation

435 methods during RC healing. Lastly, direct clinical implications for treatment may be difficult to
436 determine from this small animal model. Currently, little is known about the biological mechanisms
437 underlying the RC healing response.⁶ Therefore, basic research on these mechanisms may provide
438 clues in order to determine the safety, effect, dose, and method of administration in larger models.
439 Ultimately, human clinical trials are also needed to translate these findings to clinical use.

440 It is not known whether FGF-2 can selectively facilitate the growth of undifferentiated tenogenic
441 cells of a certain mesenchymal lineage. However, the enhanced expression of *Scx* and *Tnmd* genes in
442 the mid-to-late stages indicates that a certain microenvironment favorable for the growth response of
443 tenogenic progenitor cells to FGF-2 may be provided in the present RC healing model. Interestingly,
444 the transient upregulation of *Sox9* detected in the FGF-2-treated group at 4 weeks postoperatively
445 may indicate that a possible occurrence of *Sox9* and *Scx* double-positive tenogenic progenitors
446 contributes to enthesis formation during development.²⁰ Thus, further analysis of the cellular
447 components and the microenvironment at the RC repair sites will help develop clinical strategies for
448 inducing a specific regenerative healing response in RC injuries.

449

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526

527 **Figure legends**

528 **Figure 1:** Photomicrographs of the specimens at 2, 6 and 12 weeks postoperatively (A–F: control;
529 G–L: FGF-2-treated). The hematoxylin and eosin-stained sections (A–C, G–I) and picrosirius-red
530 stained sections under polarized light (D–F, J–L). The boxed areas in A–C and G–I are shown at a
531 higher magnification in a–c and g–i, respectively. Bars, 200 μ m.

532

533 **Figure 2:** Immunostaining for the proliferating cell nuclear antigen (PCNA) at 2 and 6 weeks
534 postoperatively (A, B: control; C, D: FGF-2-treated). The sections are counterstained with
535 hematoxylin. Bars, 100 μ m.

536 The percentage of PCNA-positive cells in the region of interest at the insertion site during rotator
537 cuff healing (E). Bar graphs, the mean for each group; error bars, one standard deviation. * $P < .05$, **
538 $P < .01$.

539

540 **Figure 3:** The gene expression levels of *Scleraxis* (A) and *Tenomodulin* (B) in the healing tissues.
541 The target genes are normalized to *18S ribosomal RNA* expression and are further normalized to the
542 intact supraspinatus tendon tissues that are equal to 1. Bar graphs, the mean for each group; error
543 bars, one standard deviation. * $P < .05$, ** $P < .01$.

544

545 **Figure 4:** *In situ* hybridization of *Scleraxis* and *Tenomodulin* at the tendon mid-substance (A–F,

M-R) and the insertion site (G-L, S-X) at 2 and 8 weeks postoperatively (A-L: control; M-X: FGF-2-treated). Sense, the negative control using a sense labeled probe. Arrows, the hybridization signals. Bars, 100 μ m.

549

Figure 5: Picrosirius-red-stained sections under polarized light (A) and *in situ* hybridization of *Tenomodulin* (B) for the FGF-2-treated group at 8 weeks postoperatively. Arrows, the hybridization signals. Bars, 200 μ m. Schematic illustration of the segmented area in healing tissues (C). (t, tendon areas; s, suture areas; i, insertion areas; a, articular side; m, middle layer; b, bursal side). Correlation between collagen fiber orientation and the expression levels of *Scx* mRNA (D), or *Tnmd* mRNA (E) for each area in both groups, respectively. Gray scale percentages are relative values compared to six intact specimens. The correlation coefficient (ρ) and *P*-value were obtained by Spearman's correlation analysis.

558

Figure 6: The gene expression levels of *Sox9* in the healing tissues. The target genes are normalized to *18S ribosomal RNA* expression and are further normalized to the intact supraspinatus tendon tissues that are equal to 1. Bar graphs, the mean for each group; error bars, one standard deviation. * $P < .05$, ** $P < .01$.

563

Figure 7: Immunostaining for *Sox9* at 2, 4, 6, and 12 weeks postoperatively (A-D: control; E-H:

565 FGF-2-treated). The percentage of Sox9-positive cells in the region of interest at the insertion site

566 (G). The sections are counterstained with hematoxylin. Bars, 50 μ m. Bar graphs, the mean for each

567 group; the error bars, one standard deviation. * $P < .05$, ** $P < .01$.

568

569 **Figure 8:** Immunostaining for mesenchymal stem cell-related surface antigens CD105 (A, F), CD90

570 (B, G), and CD73 (C, H) and hematopoietic cell antigen CD45 (D, I) and CD34 (E, J) at the insertion

571 site at 2 weeks postoperatively. A–E (control) and F–J (FGF-2-treated) are serial sections

572 counterstained with hematoxylin. Bars, 50 μ m.

573

574 Table 1

575

576

TABLE 1
The Histological Scoring System

Characteristics	Score
Cellularity (%) ^a	
> 400	1
300–400	2
200–300	3
< 200	4
Vascularity (bv/low PF) ^b	
> 15	1
10–15	2
6–10	3
< 6	4
Collagen fiber orientation (%) ^c	
< 25	1
25–50	2
50–75	3
> 75	4

^a Number of cells per region of interest from each section; percentages represent relative values compared to the values from normal tendon-to-bone sections (n = 6), which were set at 100%.

^b Number of blood vessels per low PF (100-fold magnification) from each section; bv, blood vessel; PF, power field.

^c Gray scale per region of interest from each section as measured using Image J, percentages represent relative values compared to the values from normal tendon-to-bone sections (n = 6), which were set at 100%.

577 Table 2

578

579

TABLE 2
The Summary of The Biomechanical Testing^a

Time point group	Ultimate load-to-failure (N)	Stiffness (N/mm)	Cross-sectional area (mm ²)	Ultimate stress-to-failure (N/mm ²)
6 weeks				
Control (n = 9)	7.92 ± 2.54	3.43 ± 1.24	3.53 ± 0.51	2.34 ± 0.93
FGF-2 (n = 9)	15.1 ± 3.32	7.16 ± 3.28	3.56 ± 0.35	4.24 ± 0.96
<i>P</i> value	.009	.005	.627	.001
12 weeks				
Control (n = 9)	13.8 ± 4.69 ^b	6.83 ± 1.25 ^b	3.53 ± 0.26	3.88 ± 1.12 ^b
FGF-2 (n = 9)	23.8 ± 4.61 ^b	8.98 ± 3.58	3.42 ± 0.28	6.92 ± 1.10 ^b
<i>P</i> value	.003	.310	.354	.003
Normal control (n = 9)	42.0 ± 5.56	27.0 ± 2.85	2.09 ± 0.13	19.7 ± 2.44

^aAll values are the mean ± standard deviation.

^bSignificantly different between time points within group ($P < .05$).

*Significantly different between groups at the same time point ($P < .01$).

580 Table 3

581

582

TABLE 3
Summary of The Histological Assessment ^a

Time point Treatment	Cellularity ^b (%)	Vascularity ^c (bv/ low PF)	Collagen fiber orientation ^d (%)	Total score ^e
2 weeks				
Control (n = 6)	552 ±72.5	35.7 ±8.4	17.9 ±2.5	3.0 ±0.0
FGF-2 (n = 6)	604 ±98.3	44.7 ±8.7	21.6 ±3.7	3.2 ±0.4
<i>P</i> value	.423	.109	.078	.631
4 weeks				
Control (n = 6)	448 ±56.9	31.5 ±3.4	41.4 ±10.9 ^f	4.0 ±0.6
FGF-2 (n = 6)	503 ±30.8	29.9 ±9.2 ^f	56.0 ±3.3 ^f	4.8 ±0.4
<i>P</i> value	.078	.689	.007	.045
6 weeks				
Control (n = 6)	447 ±49.1	22.7 ±5.5 ^f	57.4 ±5.3 ^f	5.2 ±0.4 ^{f,g}
FGF-2 (n = 6)	377 ±41.2 ^{f,g}	18.4 ±5.7 ^f	68.1 ±4.8 ^{f,g}	6.3 ±1.0 ^f
<i>P</i> value	.025	.298	.025	.037
8 weeks				
Control (n = 6)	389 ±41.2 ^f	21.6 ±6.6 ^f	62.5 ±4.6 ^{f,h}	5.8 ±0.4 ^{e,f}
FGF-2 (n = 6)	338 ±59.0 ^{e,f}	11.4 ±4.7 ^{f,g}	73.4 ±8.1 ^{f,g}	8.0 ±1.7 ^{e,f}
<i>P</i> value	.128	.020	.025	.013
12 weeks				
Control (n = 6)	350 ±57.2 ^e	13.6 ±3.3 ^{f,g}	70.8 ±6.4 ^{f,h}	7.2 ±1.0 ^{f,i}
FGF-2 (n = 6)	288 ±52.5 ^{f,g}	8.1 ±2.3 ^{f,g}	81.9 ±4.5 ^{f,h}	9.3 ±1.2 ^{f,h}
<i>P</i> value	.109	.013	.025	.013

^aAll values are the mean ± standard deviation.

^b Number of cells per region of interest from each section; percentages represent relative values compared to the values from normal tendon-to-bone sections (837 ± 86.3 cells/mm², n = 6), which were set to 100%.

^c Number of blood vessels per low PF (100-fold magnification) from each section; bv, blood vessel; PF, power field.

^d Gray scale per region of interest from each section as measured using Image J; percentages represent relative values compared to the values from normal tendon-to-bone sections (191.0 ± 17.5 gray scale, n = 6), which were set to 100%.

^e Scores represent a total score of three parameters including cellularity, vascularity and collagen fiber orientation. A perfect score in this scoring system is 12 points.

^{f,i} Significantly different within group compared to 2(f), 4(g), 6(h), and 8 weeks(i), respectively (*P* < .05).

*Significantly different between groups at the same time point (*P* < .05).

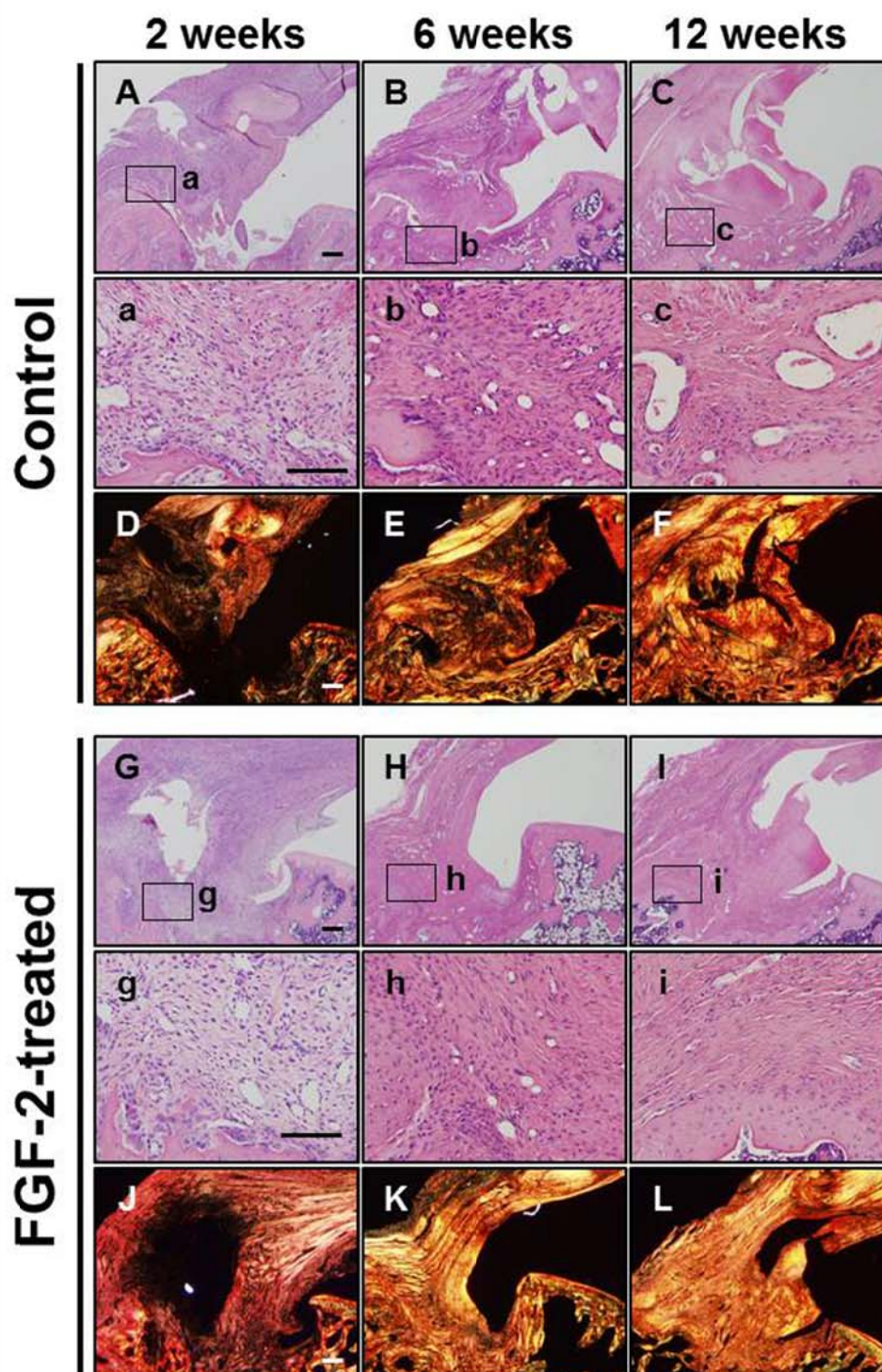


Figure 1. Photomicrographs of the specimens at 2, 6 and 12 weeks postoperatively (A–F: control; G–L: FGF-2-treated). The hematoxylin and eosin-stained sections (A–C, G–I) and picrosirius-red stained sections under polarized light (D–F, J–L). The boxed areas in A–C and G–I are shown at a higher magnification in a–c and g–i, respectively. Bars, 200 μ m.

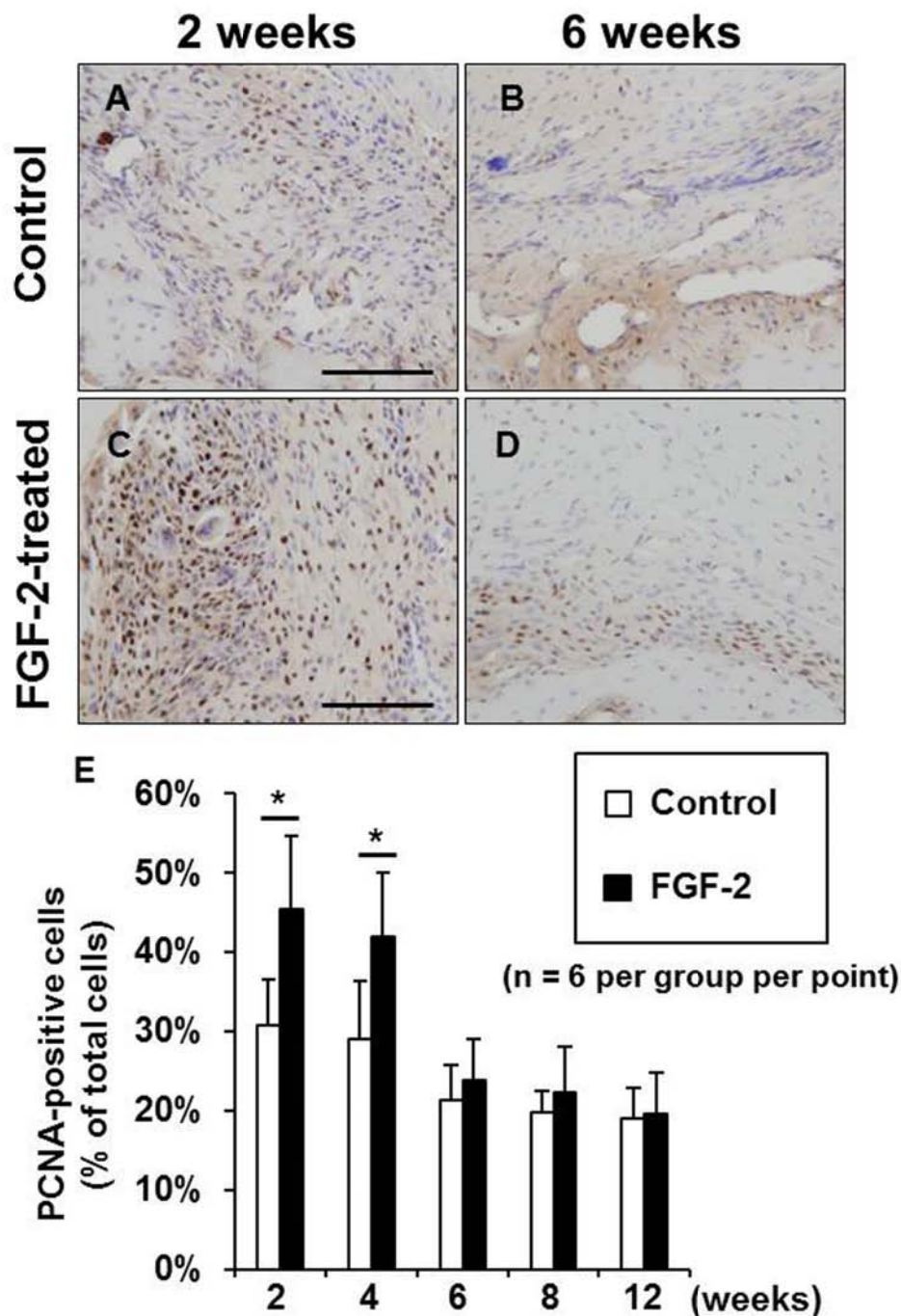


Figure 2. Immunostaining for the proliferating cell nuclear antigen (PCNA) at 2 and 6 weeks postoperatively (A, B: control; C, D: FGF-2-treated). The sections are counterstained with hematoxylin. Bars, 100 μm.

The percentage of PCNA-positive cells in the region of interest at the insertion site during rotator cuff healing (E). Bar graphs, the mean for each group; error bars, one standard deviation. * $P < .05$, ** $P < .01$.

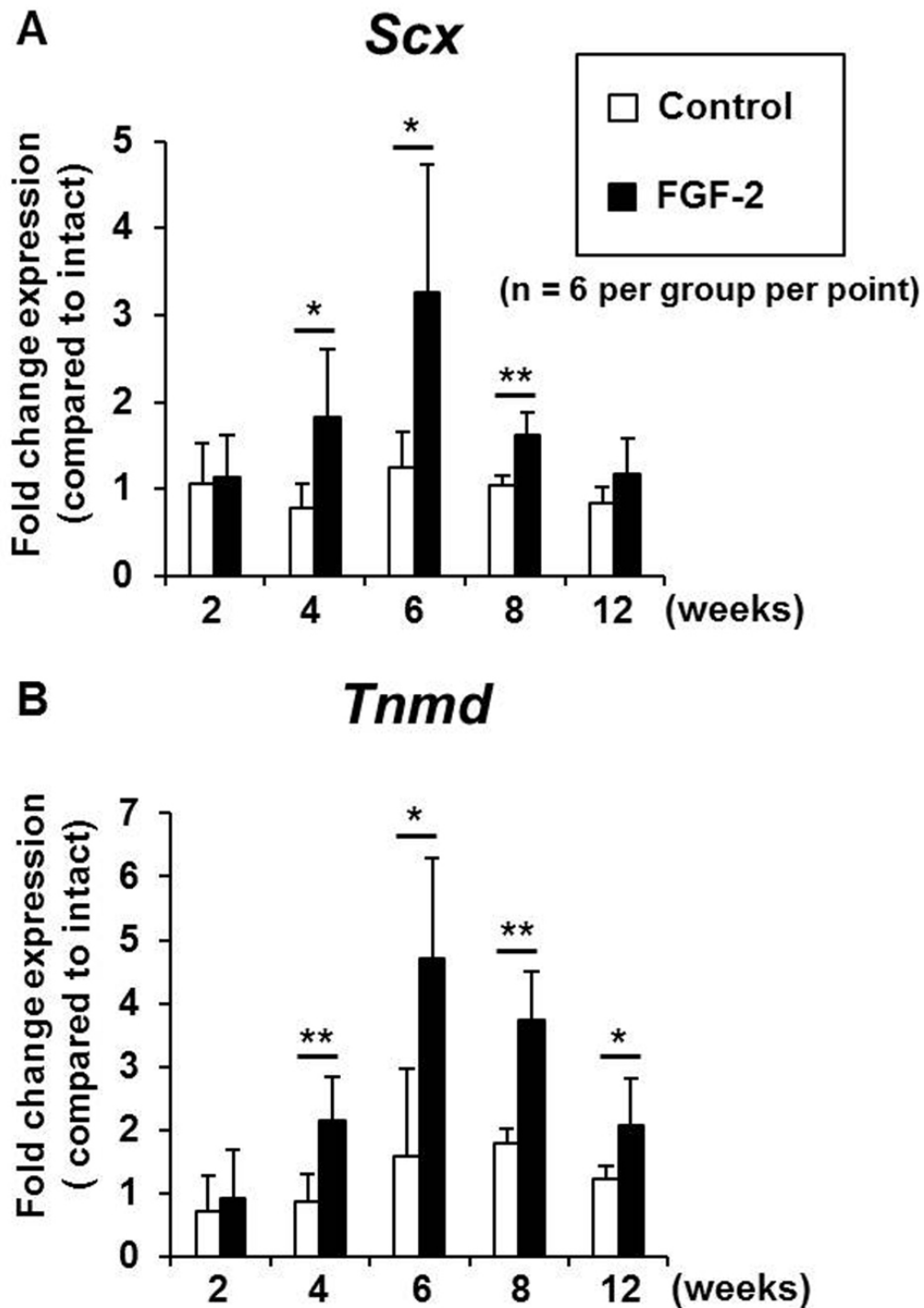


Figure 3. The gene expression levels of *Scleraxis* (A) and *Tenomodulin* (B) in the healing tissues. The target genes are normalized to *18S ribosomal RNA* expression and are further normalized to the intact supraspinatus tendon tissues that are equal to 1. Bar graphs, the mean for each group; error bars, one standard deviation. * $P < .05$, ** $P < .01$.

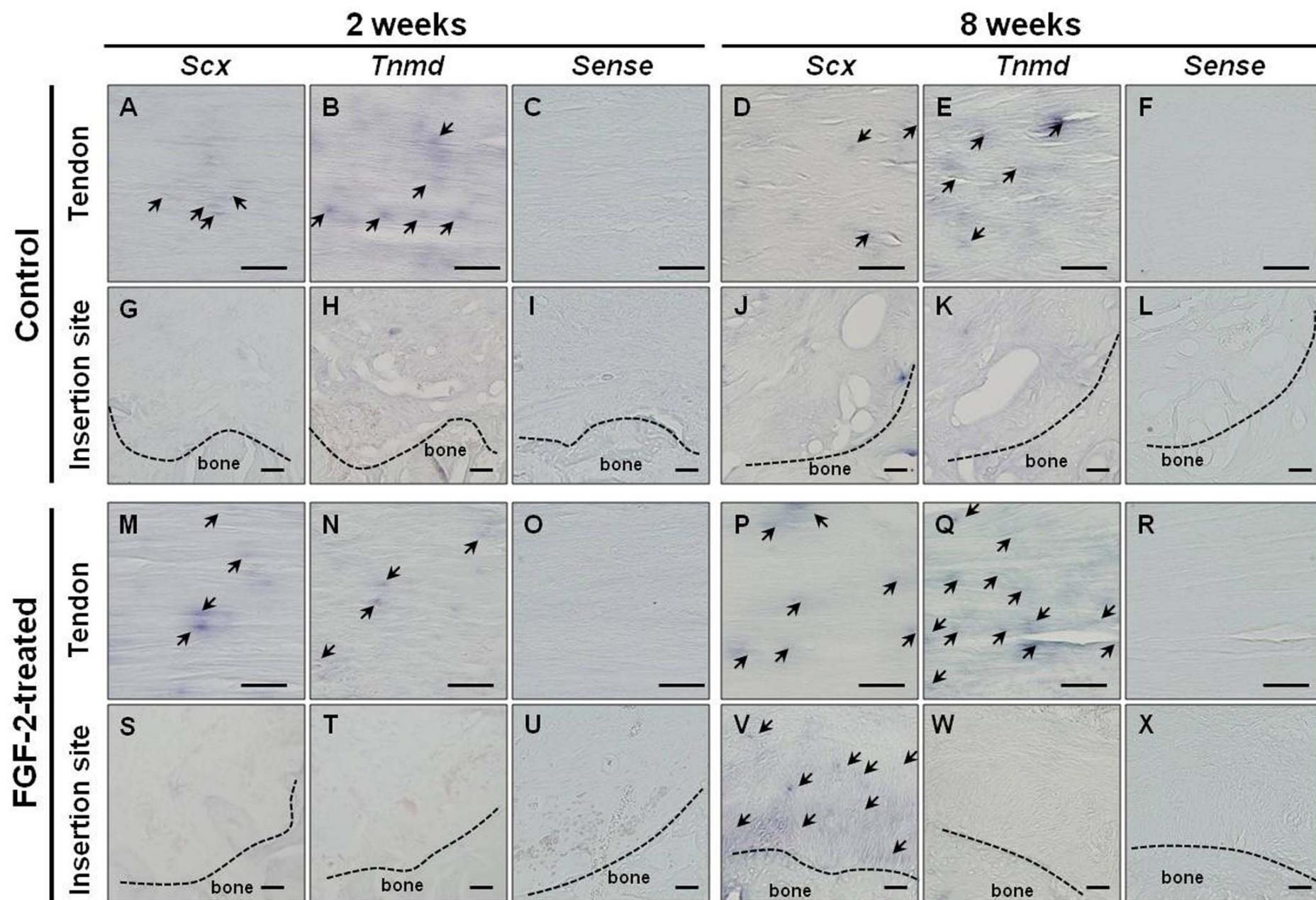


Figure 4. *In situ* hybridization of *Scleraxis* and *Tenomodulin* at the tendon mid-substance (A–F, M–R) and the insertion site (G–L, S–X) at 2 and 8 weeks postoperatively (A–L: control; M–X: FGF-2-treated). Sense, the negative control using a sense labeled probe. Arrows, the hybridization signals. Bars, 100 μ m.

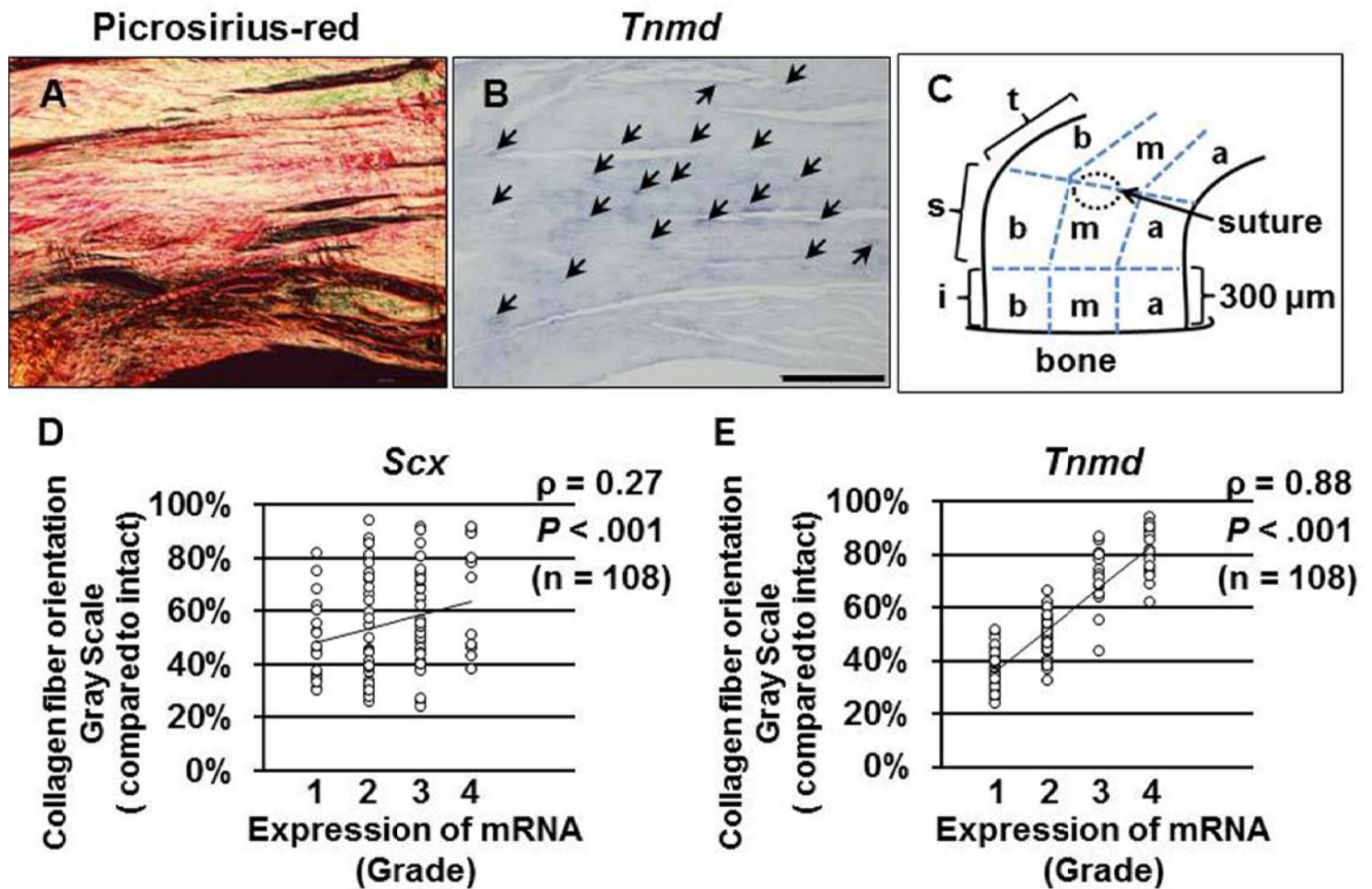


Figure 5. Picrosirius-red-stained sections under polarized light (A) and *in situ* hybridization of *Tenomodulin* (B) for the FGF-2-treated group at 8 weeks postoperatively. Arrows, the hybridization signals. Bars, 200 μm. Schematic illustration of the segmented area in healing tissues (C). (t, tendon areas; s, suture areas; i, insertion areas; a, articular side; m, middle layer; b, bursal side). Correlation between collagen fiber orientation and the expression levels of *Scx* mRNA (D), or *Tnmd* mRNA (E) for each area in both groups, respectively. Gray scale percentages are relative values compared to six intact specimens. The correlation coefficient (ρ) and *P*-value were obtained by Spearman's correlation analysis.

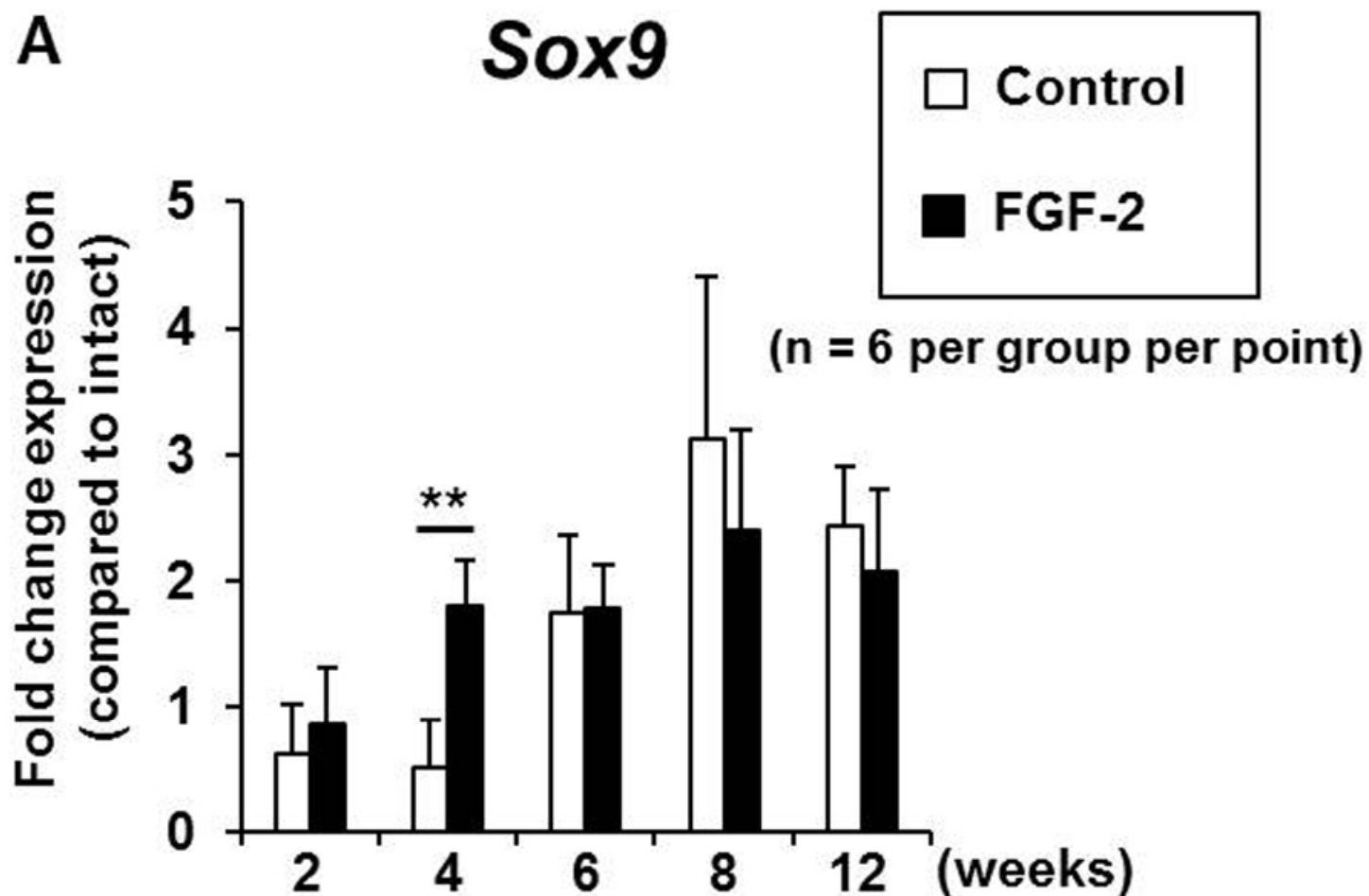


Figure 6. The gene expression levels of Sox9 in the healing tissues. The target genes are normalized to 18S *ribosomal RNA* expression and are further normalized to the intact supraspinatus tendon tissues that are equal to 1. Bar graphs, the mean for each group; error bars, one standard deviation. * $P < .05$, ** $P < .01$.

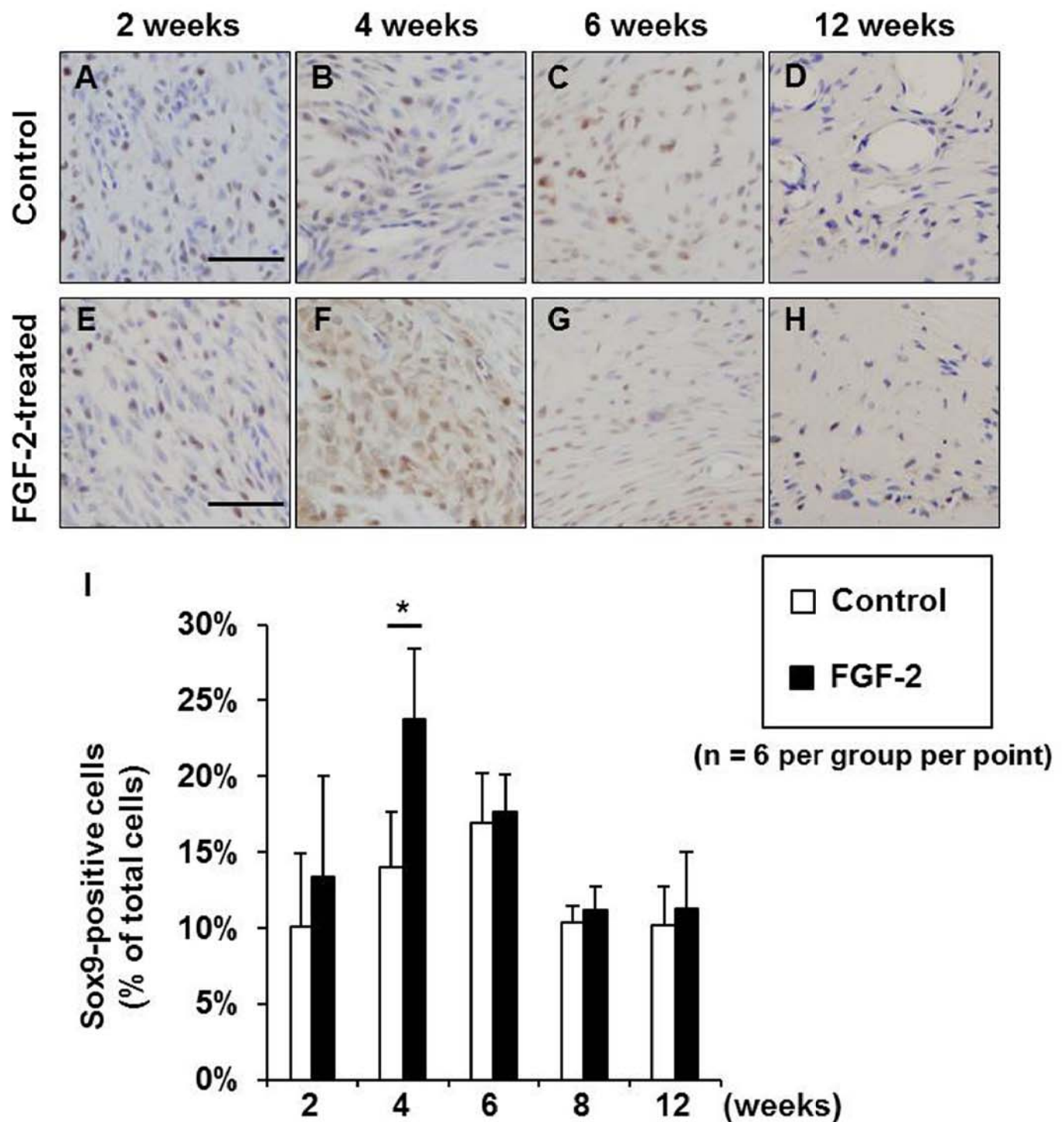


Figure 7. Immunostaining for Sox9 at 2, 4, 6, and 12 weeks postoperatively (A–D: control; E–H: FGF-2-treated). The percentage of Sox9-positive cells in the region of interest at the insertion site (G). The sections are counterstained with hematoxylin. Bars, 50 μ m. Bar graphs, the mean for each group; the error bars, one standard deviation. * $P < .05$, ** $P < .01$.

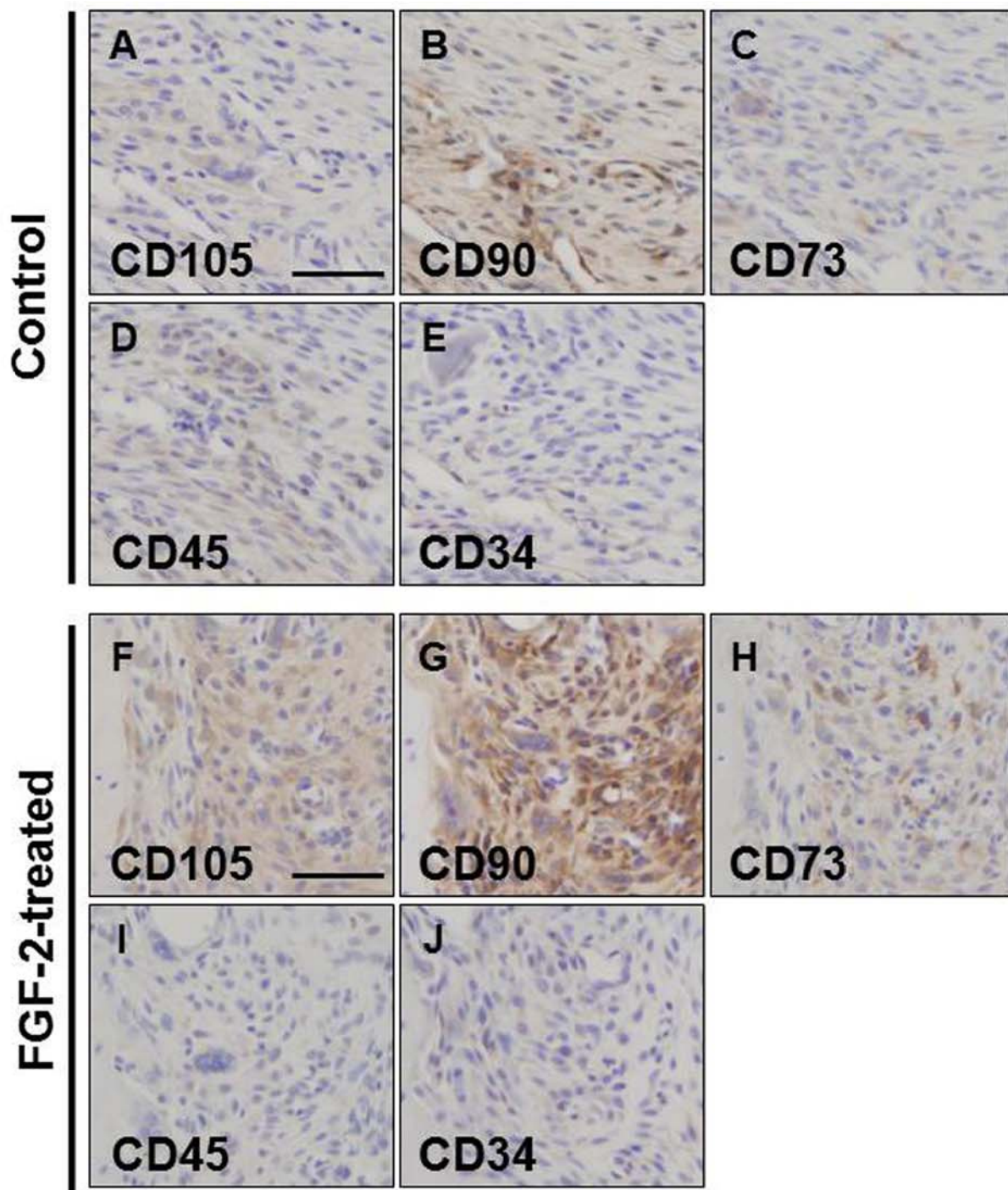


Figure 8. Immunostaining for mesenchymal stem cell-related surface antigens CD105 (A, F), CD90 (B, G), and CD73 (C, H) and hematopoietic cell antigen CD45 (D, I) and CD34 (E, J) at the insertion site at 2 weeks postoperatively. A–E (control) and F–J (FGF-2-treated) are serial sections counterstained with hematoxylin. Bars, 50 μ m.